Overexpression of the Na-K-ATPase \( \alpha_2 \)-subunit improves lung liquid clearance during ventilation-induced lung injury

Yochai Adir,1,2 Lynn C. Welch,1 Vidas Dumasius,1 Phillip Factor,3 Jacob I. Sznajder,1 and Karen M. Ridge1,4

1Division of Pulmonary and Critical Care Medicine, Northwestern University Feinberg School of Medicine, Chicago, Illinois; 2Division of Pulmonary Medicine, Carmel Medical Center, Rappaport Family Institute for Research in the Medical Sciences, Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel; 3Department of Medicine, Columbia University College of Physicians and Surgeons, New York, New York; and 4Jesse Brown Veterans Administration Medical Center-Lakeside Division, Chicago, Illinois

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Mechanical ventilation with high tidal volumes (HVT) impairs lung liquid clearance (LLC) and downregulates alveolar epithelial Na-K-ATPase. We have previously reported that the Na-K-ATPase \( \alpha_2 \)-subunit contributes to LLC in normal rat lungs. Here we tested whether overexpression of Na-K-ATPase \( \alpha_2 \)-subunit in the alveolar epithelium would increase clearance in a HVT model of lung injury. We infected rat lungs with a replication-incompetent adenovirus that expresses Na-K-ATPase \( \alpha_2 \)-subunit gene (Ad\( \alpha_2 \)) 7 days before HVT mechanical ventilation. HVT ventilation decreased LLC by 50% in untreated, sham, and Adnull-infected rats. Overexpression of Na-K-ATPase \( \alpha_2 \)-subunit prevented the decrease in clearance caused by HVT and was associated with significant increases in Na-K-ATPase \( \alpha_2 \) protein abundance and activity in peripheral lung basolateral membrane fractions. Ouabain at \( 10^{-5} \) M, a concentration that inhibits the \( \alpha_2 \) but not the Na-K-ATPase \( \alpha_1 \), decreased LLC in Ad\( \alpha_2 \)-infected rats to the same level as sham and Adnull-infected lungs, suggesting that the increased clearance in Ad\( \alpha_2 \) lungs was due to Na-K-ATPase \( \alpha_2 \) expression and activity. In summary, we provide evidence that augmentation of the Na-K-ATPase \( \alpha_2 \)-subunit, via gene transfer, may accelerate LLC in the injured lung.

acute respiratory distress syndrome; acute lung injury; experimental models; gene therapy

CLEARANCE OF PULMONARY EDEMA occurs via vectorial Na\( ^+ \) transport where water follows the Na\( ^+ \) gradients isosmotically. Na\( ^+ \) enters the alveolar epithelial cells mainly via apical amiloride-sensitive sodium channels and is extruded by the basolaterally located Na-K-ATPases (3, 8, 20, 21, 27, 30, 31). The Na-K-ATPase is a heteromeric enzyme composed of an \( \alpha \) and \( \beta \)-subunit. The \( \alpha \)-subunit is the catalytic component of the enzyme containing the binding site for ATP as well as the receptor site for cardiac glycosides (22, 26). Four \( \alpha \)-isoforms have been identified, each with a unique tissue distribution (28). The Na-K-ATPase \( \alpha_1 \)- and \( \alpha_2 \)-subunits have been reported in lung (25, 29). The smaller \( \beta \)-subunit is a glycosylated transmembrane molecule that controls \( \alpha-/\beta \)-assem-

bly and insertion of the Na-K-ATPase into the plasma membrane (29, 31).

In human lungs, the alveolar surface area available for fluid reabsorption and gas exchange is \( \sim 100 \) m\(^2 \) and is composed of alveolar epithelial type I (ATI) and type II (ATII) cells (7). ATII cells cover 2–5%, whereas ATI cells cover \( \sim 95\% \) of the surface area (7). Previous studies have shown that ATII cells are the main site for active sodium transport where ATI cells lack the Na-K-ATPase and serve as a barrier with no part in fluid clearance (13). However, recent reports demonstrated that ATI cells express the Na-K-ATPase \( \alpha_1 \)-, \( \alpha_2 \)-, and \( \beta_2 \)-isoforms and epithelial sodium channels (4, 15, 24), and, more importantly, ATII cells have a significant role in lung liquid clearance. Ridge et al. (24) reported that the Na-K-ATPase \( \alpha_2 \)-subunit is responsible for \( \sim 60\% \) of basal lung liquid clearance, and \( \sim 80\% \) of the catecholamine-mediated increase in clearance occurs via upregulation of the Na-K-ATPase \( \alpha_2 \) in ATII cells.

Mechanical ventilation is used in the care of patients with acute respiratory failure; however, ventilation with high tidal volumes (HVT) may have deleterious effects and cause lung injury (1, 6, 32). It has been demonstrated that mechanical ventilation with HVT increases microvascular filtration coefficient in isolated lungs, produces pulmonary edema in intact animals, and also decreases alveolar epithelial Na\( ^+ \)-transport, thus impairing lung liquid clearance (1, 9, 32, 33).

Recently, we have demonstrated that adenoviral-mediated gene transfer results in overexpression of Na-K-ATPase subunits in the alveolar epithelium (12). Overexpression of the Na-K-ATPase \( \alpha_2 \)-subunit was associated with \( \sim 250\% \) increase in lung liquid clearance (LLC) in normal rats (24), and a recent study suggests that Na-K-ATPase \( \beta_1 \)-subunit gene overexpression in the alveolar epithelium increases Na-K-ATPase function and LLC in a model of HVT (10). Therefore, we hypothesized that Na-K-ATPase \( \alpha_2 \)-subunit (which is normally expressed in ATI cells) overexpression in the alveolar epithelium could positively affect LLC during HVT-induced lung injury in rats. To test this hypothesis, we infected rat lungs with a replication-incompetent adenovirus that expresses a rat Na-K-ATPase \( \alpha_2 \)-subunit gene (Ad\( \alpha_2 \)) compared with sham and Adnull-infected controls 7 days before HVT mechanical ventilation and measurement of LLC.

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METHODS

Adenovirus delivery. Replication-incompetent (E1a−/E3−) human type 5 adenoviruses containing expression cassettes with a human immediate/early CMV promoter/enhancer and a rat Na-K-ATPase α2-subunit cDNA (Adα2) or no cDNA (AdNull) were constructed, propagated, purified, and titered as previously described (10). The use of animals for this study was approved by the Northwestern University Institutional Animal Care and Use Committee, according to the NIH guidelines. All animals were provided food and water ad libitum and were maintained on a 12:12-h light-dark cycle. Rats were anesthetized with 40 mg/kg pentobarbital intraperitoneally and orally intubated with a 14-g plastic catheter before adenoviral infection (24). Three experimental groups were studied: sham-surfactant, AdNull, and Adα2. A mixture of adenovirus in a 50% surfactant/50% dialysis buffer vehicle was administrated in four aliquots of 200 μl. Rats were rotated 90° between instillations given at 5-min intervals. Immediately before instillation, a forced exhalation was achieved by circumferential compression of the thorax. Compression was relinquished after endotracheal instillation of 200 μl of virus/vehicle followed by 800 μl of air. Rats were allowed to recover before exudation. Infected animals were maintained in separate isolator cages for 7 days before conducting experimental protocols.

Measurement of LLC. The isolated, fluid-filled, perfused lung preparation was performed immediately following HVȚ ventilation as previously described (1, 2, 10, 16). Changes in concentration of Evans blue-tagged albumin instilled into the air space were used to estimate the volume of fluid removed from the alveolar air space. The total unidirectional flux of Na+ from the alveolar space (i.e., active transport and passive movement) was calculated from the rate of loss of 22Na+ from the air spaces. Passive Na+ flux was calculated by subtracting the active Na+ flux (calculated from the rate of net fluid clearance) from total Na+ flux (27). Similarly, the flux of mannitol was calculated from the rate of loss of [1H]mannitol from the air spaces (27). Albumin flux from the pulmonary circulation into the alveolar space was determined from the fraction of FITC-labeled albumin, placed in the perfusate that appeared in the alveolar instillate during the experimental protocol.

Experimental protocols. Six experimental groups of rats were used in this study: noninfected/noninfected (n = 6), Adα2-infected nonventedilated (n = 6), noninfected HVȚ (n = 6), Adα2-infected + HVȚ (n = 6), AdNull + HVȚ (n = 4), and sham + HVȚ (n = 4). LLC was measured for two consecutive hours. In the first hour, LLC was expressed as percentage of the instillate volume. In the second hour, LLC was expressed as percentage of the instillate volume.

RESULTS

LLC. LLC was increased ~70% in rats infected with Adα2 compared with control (n = 6), Adnull-infected (n = 4), or sham-infected rats (n = 4) (Fig. 1). In rats ventilated with HVȚ, and basolateral membranes (BLM) as previously described (12, 17). Briefly, cell lysates were prepared by addition of lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 25 mM sodium pyrophosphate, 1 mM Na3VO4, 1 μM leupeptin, 1 mM PMSF) and centrifugation at 14,000 g to eliminate the insoluble material. BLM were prepared using Percoll gradient centrifugation as previously described (12, 17). Peripheral lung tissue was homogenized in a buffer containing 300 mM mannitol in 12 mM Tris-HEPES, pH 7.6, and protease inhibitors as described above and centrifuged twice to discard the nuclear and mitochondrial pellet. Supernatant was centrifuged at 48,000 g for 30 min, and the BLM fraction was recovered after the membrane pellet was centrifuged in a 16% Percoll gradient at 48,000 g for 30 min. An equal amount of proteins from cell lysates or BLMs was resolved by 10% SDS-PAGE and analyzed by immunoblotting with specific monoclonal anti-rat Na-K-ATPase α2 (McB2, generous gift of K. Sweeney, Harvard University). The density of the bands was quantified and normalized to sham-infected controls.

Na-K-ATPase activity. Na-K-ATPase activity was determined by [32P]ATP hydrolysis as described before (18, 24). Briefly, ATII cells were plated on ice, and aliquots (~10 μg protein) were transferred to the Na-K-ATPase assay medium (final volume 100 μl) containing in mM: 50 NaCl, 5 KCl, 10 MgCl2, 1 EGTA, 50 Tris-HCl, 7 Na2ATP, and [γ-32P]ATP (specific activity 3,000 Ci/mmol) in trace amounts (3.3 nCi/μl). The samples were then incubated at 37°C for 30 min, and the reaction was terminated by addition of 700 μl of TCA/charcoal (5%/10% wt/vol) suspension and rapid cooling to 4°C. After separating the charcoal phase (12,000 g for 5 min) containing the unhydrolyzed nucleotide, the liberated 32P was counted in an aliquot (200 μl) from the supernatant. Na-K-ATPase activity was calculated as the difference between test samples (total ATPase activity) and samples assayed in the same medium, but devoid of Na+ and K+ and in the presence of 4 mM ouabain (ouabain-insensitive ATPase activity) (18, 24). Results are expressed as mean nmol Pi/μg protein/hour or triplicate measurements from three animals per group.

Statistical analysis. Data are presented as mean values ± SD. One-way analysis of variance was used when multiple comparisons were made. Differences among groups were considered significant when P value was <0.05.
LLC decreased by ~50% in noninfected control (n = 6), sham-infected (n = 4), and Adnull-infected (n = 4) rat lungs compared with nonventilated/noninfected control lungs (n = 6). Overexpression of the Na-K-ATPase α2-subunit restored the lung's ability to clear edema in rats exposed to HV_T ventilation for 40 min (from 12% to 20.4% per hour) (Fig. 1).

In rodent tissue, it is possible to distinguish physiologically between the Na-K-ATPase α1- and α2-subunits due to the large difference in their affinity for ouabain. The Na-K-ATPase α2-subunit, but not the Na-K-ATPase α1-subunit, is inhibited by 10 μM ouabain (24), whereas >1 mM ouabain is needed to inhibit the rat Na-K-ATPase α1-subunit (24). We determined the contribution of the Na-K-ATPase α2-subunit to LLC by perfusing 10 μM ouabain through the pulmonary circulation of the isolated rat lung model. In the presence of 10 μM ouabain, LLC was decreased ~60% in noninfected control, Adnull-, and sham-infected HV_T rats compared with nonventilated/noninfected control rats, suggesting that the Na-K-ATPase α2 is important in LLC (Fig. 2). Importantly, in rat lungs infected with Adα2 and subsequently ventilated with HV_T, ~80% of the increase in LLC was due to the high-affinity ouabain-sensitive Na-K-ATPase α2-subunit (Fig. 2). Alveolar epithelial permeability to small solutes (22Na\(^+\) and [3H]mannitol) increased in rats ventilated with HV_T for 40 min compared with noninfected, nonventilated control rats (Fig. 3). Evans blue dye-bound albumin instilled in the air space was not detected in the perfusate or bath compartments in any of the experimental groups. The movement of FITC-albumin from the pulmonary circulation into air space was increased in animals ventilated with HV_T (Fig. 3).

To determine whether the changes in LLC were due to changes in the expression and function of the Na-K-ATPase, we isolated BLM from peripheral lung tissue or ATII cells from HV_T-ventilated and nonventilated rats infected with Adα2, Adnull, or control, noninfected rats. As shown in Figs. 4 and 5, HV_T resulted in a significant decrease in Na-K-ATPase α1, α2, and β1 protein abundance and Na-K-ATPase activity in Adnull and control, noninfected rats compared with nonventilated, Adnull-infected and control, noninfected rats. In contrast, there was an ~57% and 49% increase in Na-K-ATPase α2 protein abundance and Na-K-ATPase activity, respectively, in nonventilated, Adα2-infected rats.

Importantly, Na-K-ATPase α2 protein abundance and Na-K-ATPase activity was maintained in HV_T-ventilated Adα2 rats compared with HV_T-ventilated Adnull-infected rats. These results suggest that overexpression of the Na-K-ATPase α2 protein maintains the Na-K-ATPase activity which then contributes to the restoration of LLC in rats exposed to HV_T ventilation.

**DISCUSSION**

Although mechanical ventilation is a life-saving procedure in patients with acute respiratory failure, it can also induce or worsen acute lung injury (1). Previous studies have shown that ventilation with HV_T can disrupt the alveolo-capillary barrier, which results in leakage of fluid and blood constituents into the alveolar spaces as well release of inflammatory mediators (1, 5, 9, 23, 32, 33). Other reports have demonstrated that mechanical ventilation with HV_T diminishes LLC. These changes were associated with decreased Na-K-ATPase activity in alveolar epithelial cells isolated from HV_T-ventilated lungs (6, 9). Recently, several studies reported that both ATII and ATII cells have a role in active sodium transport and that ATII express both the Na-K-ATPase α1 and the α2 isoforms (4, 16, 24). It has also been suggested that the Na-K-ATPase α2 contributes to the active Na\(^+\) transport and LLC, which can be further increased by stimulation of the β-adrenergic receptor or by adenovirus-mediated overexpression of the Na-K-ATPase α2-subunit in noninjured, normal rat lungs (24).

In basal conditions, the Na-K-ATPase α1 is working at ~20–50% of its maximum capacity, whereas the Na-K-ATPase α2 is working at 1/20th of its Vmax (18). A previous report demon-
Na-K-ATPase α2-SUBUNIT OVEREXPRESSION

Stratified that overexpression of the Na-K-ATPase α1 subunit in the rat epithelium did not increase Na-K-ATPase activity or LLC, however, overexpression of the Na-K-ATPase β1-subunit resulted in increased fluid clearance from rat lungs (12). Here, we report that overexpression of the Na-K-ATPase α2, which has been reported to be localized to rat ATI cells (24), plays an important role in LLC in the injured lung. We speculate that is due to the Na-K-ATPase α2-subunit’s large reserve capacity to exchanging Na⁺ and K⁺ compared with the Na-K-ATPase α1-subunit. Since the ATI cells cover more than 95% of the alveolar surface area and express key transport proteins (i.e., Na⁺ channels and Na-K-ATPase), our results support the concept that ATI cells have an active role in LLC. HV₇ causes epithelial cell injury, including ATI cell damage, leading to edema in part due to the decreased ability of ATI cells to respond to the increased need of edema resolution. In patients with adult respiratory distress syndrome, impaired ability of the lung to clear edema was associated with worse outcomes (33), which prompted us to study whether overexpression of the Na-K-ATPase α2 would upregulate alveolar Na-K-ATPase function and sustain LLC in a HV₇ model of acute lung injury previously shown to impair alveolar active Na⁺ transport (16).

In the present study, we provide evidence that overexpressing the Na-K-ATPase α2 significantly increased LLC in a model of HV₇ ventilation in rats. HV₇ reduced LLC by ~50% in untreated, sham, and Adnull-infected controls confirming that this HV₇ model impairs alveolar fluid clearance in rats. Notably, overexpression of Na-K-ATPase α2-subunit was protective in rats ventilated with HV₇ as LLC was ~350% higher than in the other HV₇ groups. Clearance in the Adα2/HV₇ lungs was 70% greater than nonventilated, uninfected controls and similar to Adα2-overexpressing nonventilated lungs (see Fig. 1).

In rodent tissues the Na-K-ATPase α1- and α2-subunits can be distinguished by their low and high affinity for ouabain, respectively (22, 25, 26). As shown in Fig. 2, ouabain at 10⁻⁵ M, a concentration that predominantly inhibits the Na-K-ATPase α2, decreased LLC in Adα2-infected lungs to the same level as sham and Adnull-infected lungs suggesting that the observed increases in LLC in Adα2 lungs are due to increased Na-K-ATPase α2 activity. Western blot analysis of BLMs isolated from the peripheral lung of HV₇ rats demonstrated that α2-subunit gene transfer significantly increased the abundance of the α2-subunit protein, whereas HV₇ significantly decreased the α1-subunit protein in all rat lungs including HV₇-Adα₂ rats. Also, we observed that α2-subunit overexpression was associated with a significant increase in Na-K-ATPase activity in BLM fractions isolated from the peripheral lung.

We have previously shown that adenoviral-mediated gene transfer can result in sustained overexpression of Na-K-ATPase in...
the lung for at least 10 days in rats, and overexpression of the Na-K-ATPase β1-subunit had beneficial effect in hyperoxic lung injury and restored LLC in the rat models of ventilator-induced lung injury and acute left atrial pressure elevation (1, 2, 10). During acute lung injury reduction of active Na+ transport, absorptive capacity and impairment of alveolar barrier function shifts transalveolar fluid balance toward air space edema accumulation which represents a significant challenge in the treatment of these patients. Adenoviral-mediated gene expression facilitates prolonged transgene expression and reduced host responses (14). High-efficient gene transfer to severely injured edematous rats lungs has been previously demonstrated (11, 19), which is encouraging for the design of gene transfer strategies to treat patients with acute lung injury.

In summary, we provide the first evidence that overexpression of the α2 Na-K-ATPase subunit has a beneficial effect on the HV ventilation-injured lung by upregulating LLC. Since the α2 Na-K-ATPase is expressed in ATI cells, which cover more than 95% of the alveolar surface area, our results support the notion that ATI cells may have a significant role in LLC, and augmentation of alveolar Na-K-ATPase function, via gene transfer, is of benefit during ventilation-induced lung injury.

GRANTS

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